

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of
Daniel V Santi et al

Application Serial No.: 10/820,975

Filed: April 7, 2004

For: SYNTHETIC GENES

:
: Confirmation No.
: 9532
:
: Group Art Unit:
: 1633
:
:
: Examiner:
: Ileana Popa
X

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. SARAH J. REISINGER

Pursuant to 37 CFR §§ 1.131 AND 1.132

I, Sarah J Reisinger, declare as follows:

1. I am an inventor in the above-identified application.
2. On the face of the above-identified application, I am listed as Sarah J. Kodumal.
3. I affirm that the date of conception of the instant invention is prior to October 3, 2003, as demonstrated by the following evidence:
 - a) pages of a presentation, attached hereto as Exhibit 1, outlines the process for combining synthon fragments (i.e., the DNA segments and/or vector segments referred to in the claims) using rational restriction site assignments;
 - b) pages of project overview presentation, attached hereto as Exhibit 2, demonstrate conception of multiple synthon assembly and dual selection markers;
 - c) pages of my notebook, attached hereto as Exhibit 3, demonstrate conception of a dual-selection marker (tet/strep). The latter may be considered as a counterselectable marker;

d) a page of the progress report, attached hereto as Exhibit 4, demonstrates conception of a possibility to perform ligations “without purifying synthon fragments, and the procedure would only require plasmid purifications after each round of synthon insertion.”

3. Additional experiments have been conducted through which actual reduction to practice of the invention have occurred. The pages from my notebook, attached hereto as Exhibit 5 describe experiments conducted in February 23-25, 2004. In these experiments, a successful four-piece ligation was performed using unpurified products of enzymatic digestion.

4. The aforementioned date of conception applies to claims 1-38 of the above-identified application, as well as to claim 39 as amended.

5. Due diligence in the instant invention was exercised by the Applicants from before October 3, 2003 through February 23-25, 2004, the date of actually reducing the invention to practice.

6. Exhibit 2 also shows unexpectedly superior results of using a two-marker vectors over one-marker vectors. Specifically, when we used one-marker vectors, 28% of the clones containing respective ligation products were false positives (i.e., did not contain the correct inserts in correct orientation). In contrast, when two-marker vectors were used (i.e., each and every clone tested had the correct insert in the correct orientation), the number of false positive clones was reduced to zero.

I, Sarah J Reisinger, hereby swear that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willfully false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued hereon.

Date: 12/1/08

Respectfully Submitted


Sarah J Reisinger

EXHIBIT 1

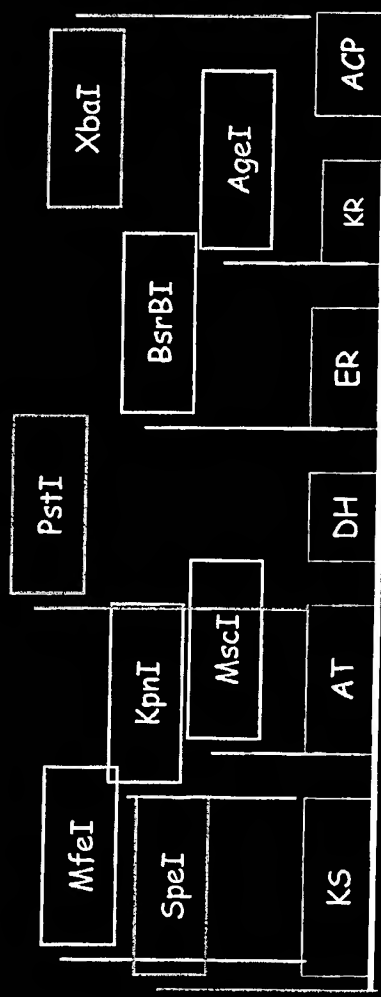
Module Gene Design

- Common restriction sites in all modules for modularity
 - flanking modules
 - flanking domains
- Restriction sites for sewing synthons together
- Intra-synthon restriction sites to correct errors in synthesis

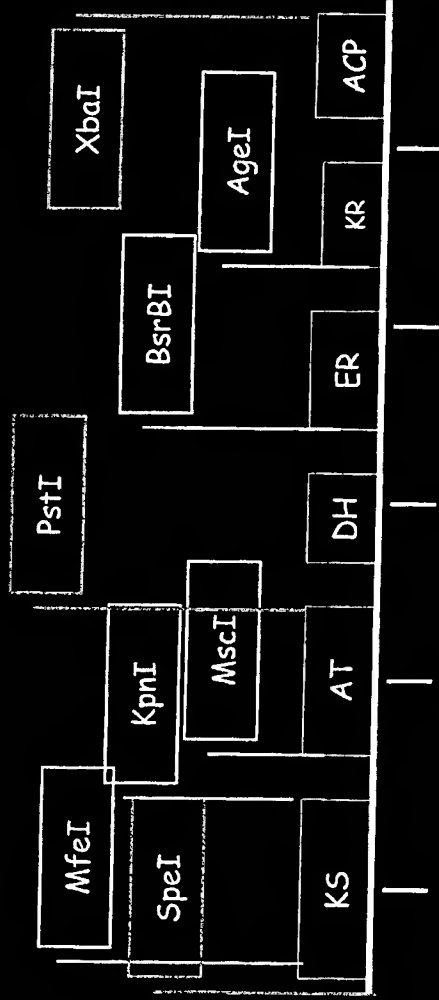
*Synthon = unit of DNA synthesized (350-750 bp);

Currently about 8-15 synthons per module

Module Gene Design



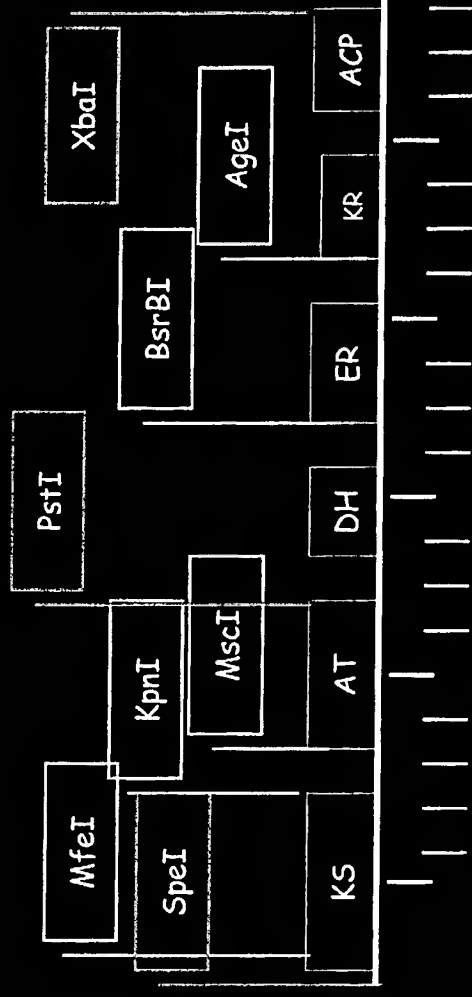
Module Gene Design



Additional sites for synthon edges



Module Gene Design



Additional intra-synthon sites for repair



EXHIBIT 2

Gene Assembly (“Synthon Sewing”)

Criteria:

Accurate

Amenable to HT

A

B

synthon

~10 plasmids containing
500 bp synthons

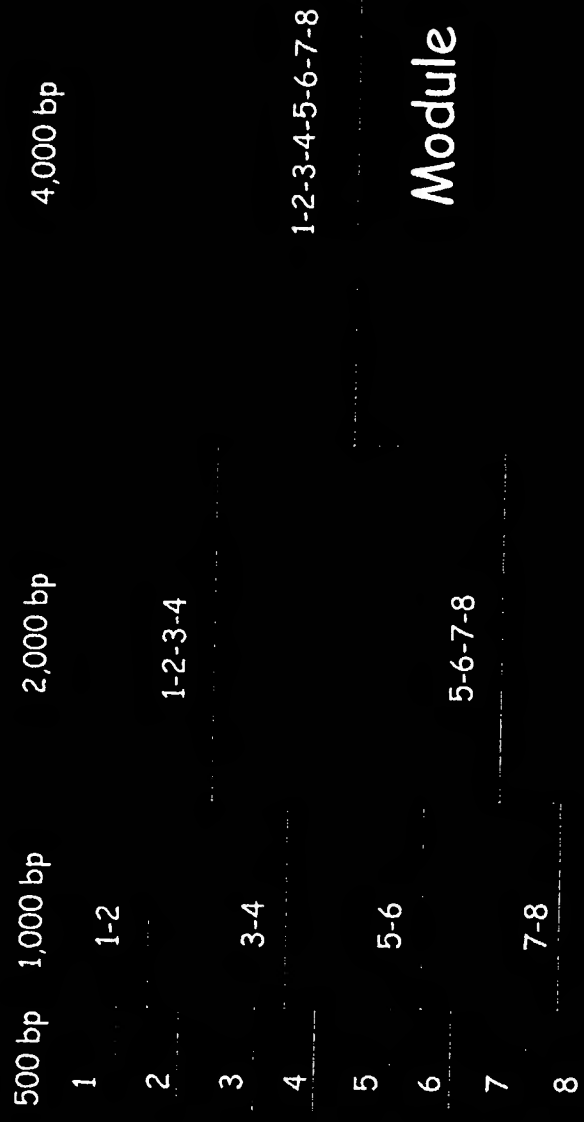


Automated stitching of synthons

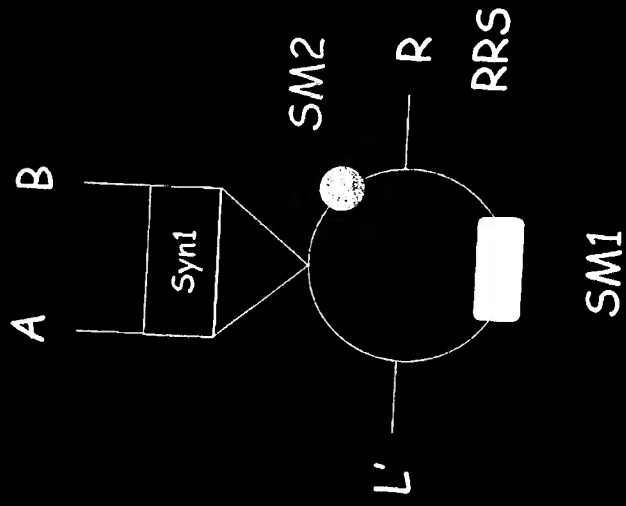


5,000 bp module

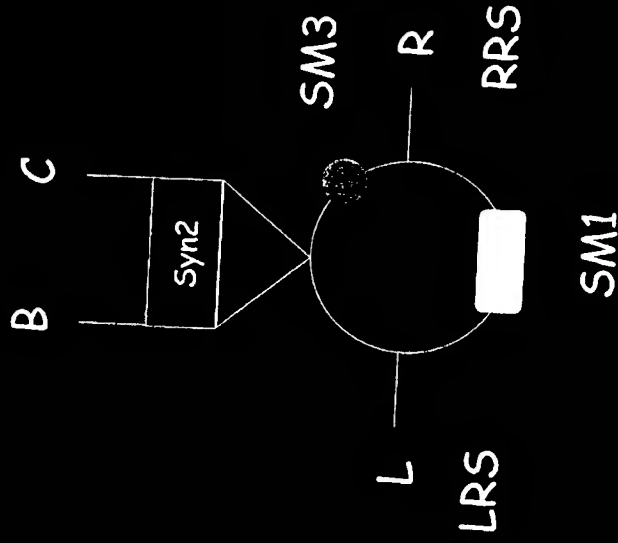
Assemble fragments into modules



Synthon Sewing



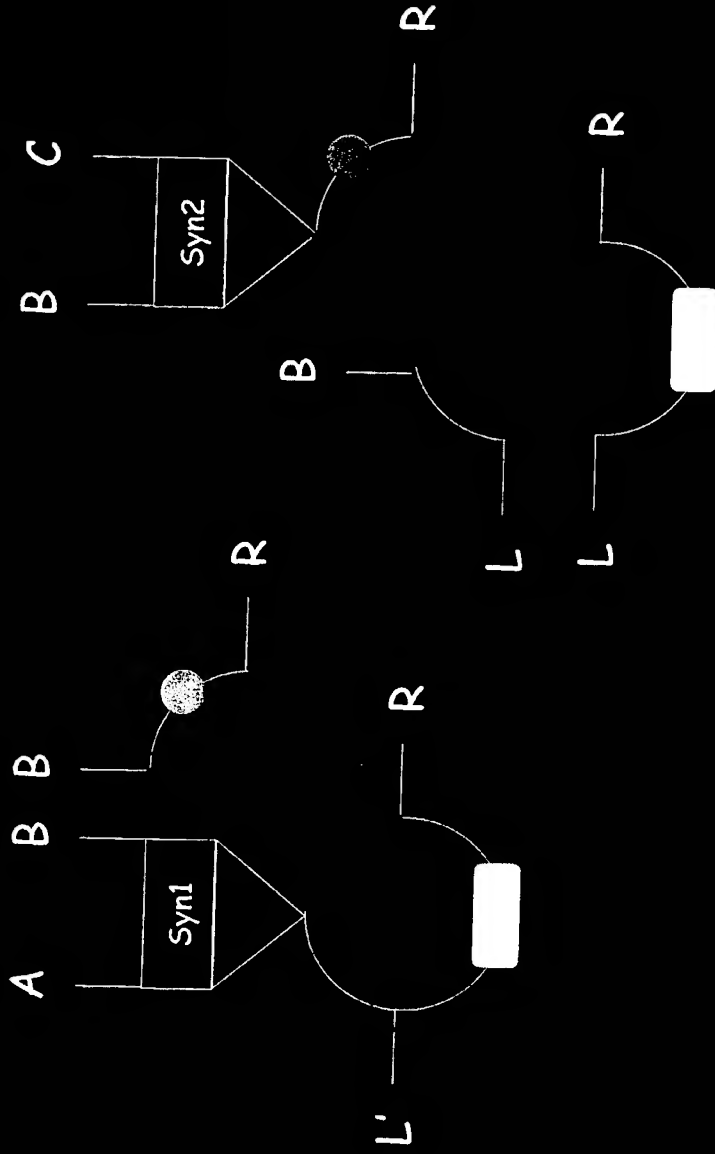
acceptor vector



donor vector

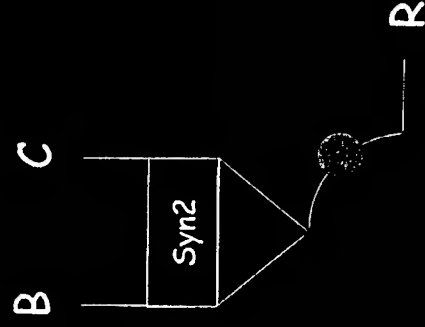
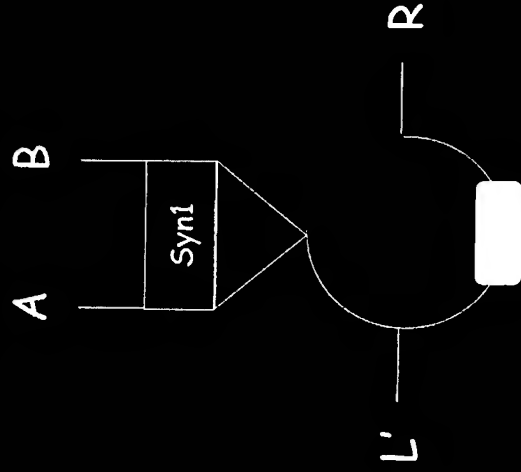
Synthon Sewing

Cut with B, R, L



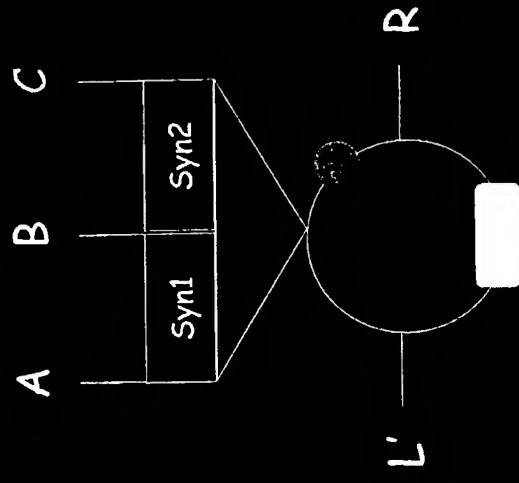
Synthon Sewing

Cut with B, R, L



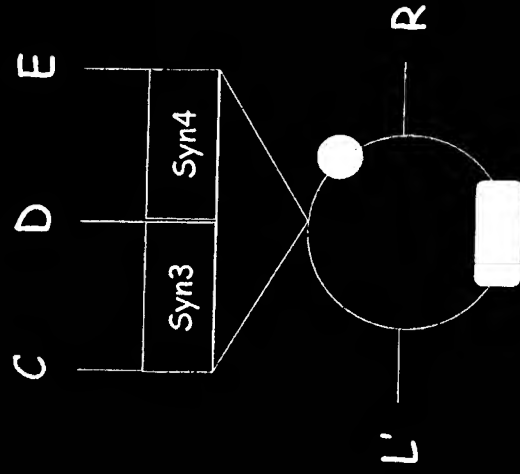
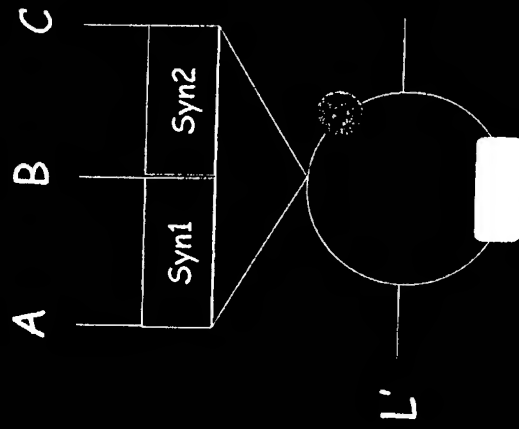
Synthon Sewing

Ligate, select, SM1 & 3

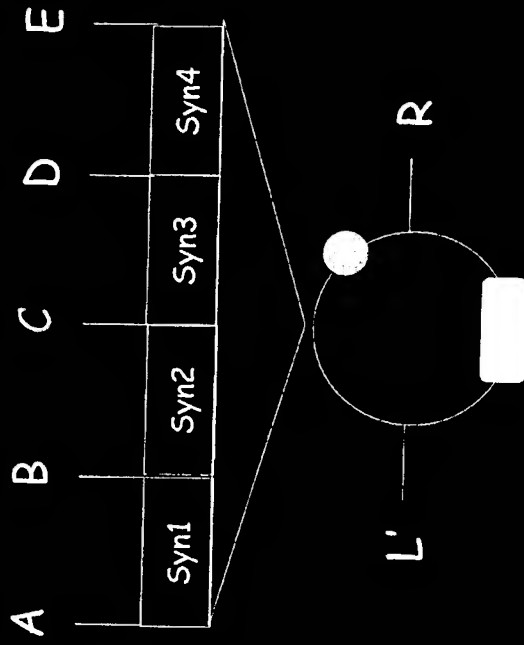


Synthon Sewing

Repeat processes with next mod pair



Synthon Sewing



Module Assembly (Synthon "Sewing")

Results

- 18 complete modules constructed
- > 120 successful ligations
- 72% of clones screened were correct

Initial Synthon Sewing Limitations

- Method requires a number of different restriction enzymes (different one for each synthon edge)
 - Not very automatable
- Presence of false positive (background) clones

New Synthon Sewing Method

- Addition of second unique selectable marker on each plasmid to reduce false positives
- Utilize Type II restriction enzymes
 - Cuts DNA outside of recognition site

5' ... G G T C T C (N)₁[^] ... 3'
3' ... C C A G A G (N)₅[^] ... 5'

- Use of different Type II enzymes that create compatible ends allows for cohesion of fragments

Results of Updated Synthon Sewing Method

- Selection scheme works extremely well
 - 100% correct in initial tests
 - Complete elimination of false positives
- Reduction in number of restriction enzymes allows for more genes to be assembled in parallel

EXHIBIT 3

From Page 121

I also designed PRIMERS for ^{synthetic} cloning vector
to amplify SM203 (neo + CAT)

Morphing Project Synthon Cloning Vector

Introduction of Second Selectable marker

1. For Chloramphenicol (cat):
-amplify using pACYC184
-linearize template using Bam HI/ Xba I

Primers:

293-122-Cm Fwd
Forward (binds 384-365): 5' AATCCTGGTGTCCCTGTTGA 3'
293-122-Cm Rev
Reverse (binds 3751-3770): 5' CTCGAGTTATTCAGGCGTAGCACCAG 3'

2. For Kanamycin (neo):
-amplify using pET26b
-linearize with Xho I/Xba I

Primers:

293-122-KAN-Fwd
Forward (binds 3726-3745): 5' TGGTATCTGCGCTCTGCTGAAG 3'
293-122-KAN-Rev
Reverse (binds 4895-4876): 5' CTCGAGTTCAGGTGGCACTTTTCGGG 3'

RED is Xho I site (R) in cloning scheme

ORDER # 6104078
CUSTOMER # 3002790
P.O. # 10600

Data Sheet



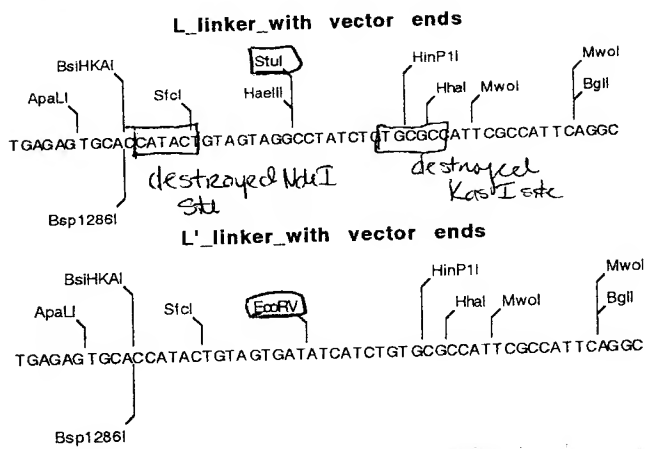
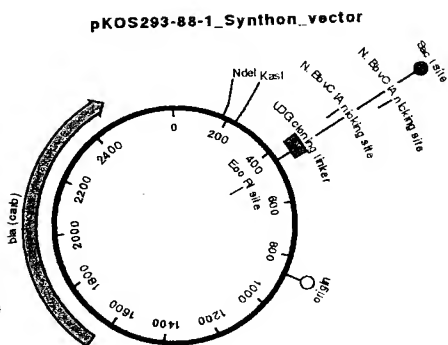
Seq #	Seq Name	Seq 5' to 3'	OD	pmol	Len	MW	µg	E260	Tm	Scale	Purif.
5300	293-122-Cm Fwd	AATCCTGGTGTCCCTGTTGA	16.14	88944.19	20	6098.98	542.47	181462.1	60.4	50 nmole	Salt Free
5301	293-122-Cm_Rev	CTCGAGTTATTCAGGCGTAGCACC AG	20.5	82357.37	26	7971.19	656.49	248915.2	67.75	50 nmole	Salt Free
5302	293-122-Kan_Fwd	TGGTATCTGCGCTCTGCTGAAG	18.03	89083.27	22	6757.4	601.97	202394.9	64.54	50 nmole	Salt Free
5303	293-122-Kan_Rev	CTCGAGTTCAGGTGGCACTTTTCG GG	21.34	89763.13	26	8009.2	718.93	237736.8	69.32	50 nmole	Salt Free

100 pmol/µL

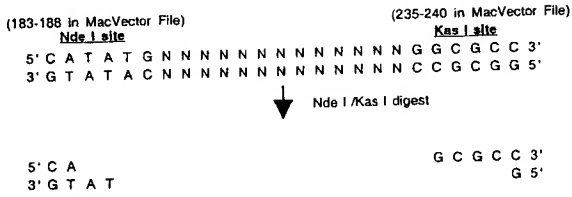
124

From Page 124

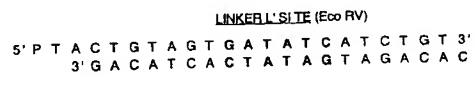
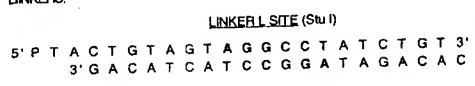
Synthon cloning vector: designed linker to destroy KasI site and insert L (StuI) or L' (EcoRV)



Synthon Cloning Vector
Linker design for L and L'
Destroys NdeI and KasI sites in process



LINKERS:



A-StuI
B-StuI
C-EcoRV
D-EcoRV

TITLE Synthon Cloning Vector

Project No. _____
Book No. 293

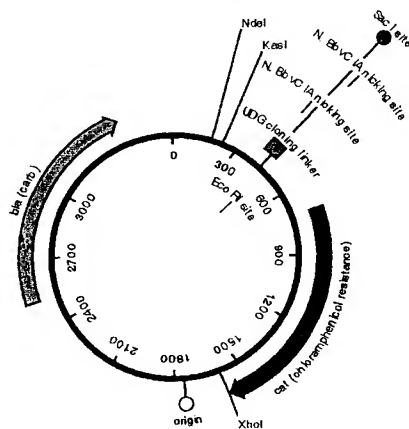
167

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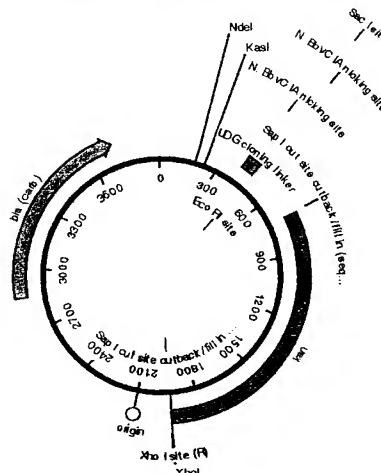
Also redesigned oligos to insert L^o L' linker.

Goal: Remove Nde I and Kas I sites from vector and insert linker containing either Stu I or Eco RV site. Stu I is blocked by overlapping dcm methylation, so no gg or cc near restriction sites. The diagrams of the vectors I am trying to insert linker into are shown, as well as a blow up of the region between Nde I and Kas I.

293-135-52_cm_carb_Synthon



293-148-18_Kan_carb_synthon



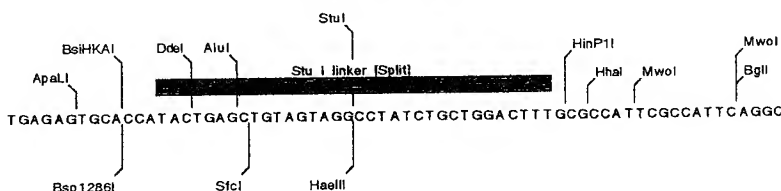
293-135-52_cm_carb_Synthon

NdeI
GAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCCCATTCGCC
200
KasI

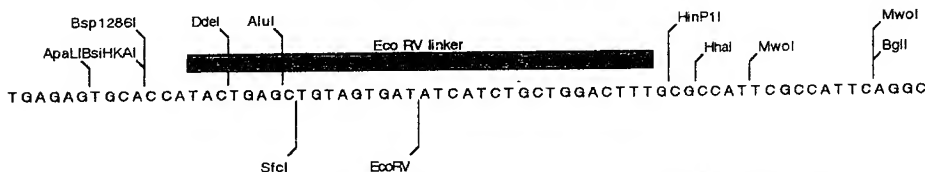
293-148-18_Kan_carb_synthon

NdeI
TGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCCCATTCGCC
200
KasI

L_linker_with vector ends



L'_linker_with vector ends



293-167-Eco RV
293-167-Eco RV_RC

5'TACTGAGCTGTAGTGATATCATCTGCTGGACTTT 3'
3' GACTCGACATCACTATAGTAGACGACCTGAAACGCG 5'

293-167-Stu I
293-167-Stu I_RC

5' TACTGAGCTGTAGTAGGCCTATCTGCTGGACTTT 3'
3' GACTCGACATCATCCGGATAGACGACCTGAAACGCG 5'

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TITLE NDG-LIC Vector improvement

Project No. _____
Book No. 399

From Page No. _____

3

Oligos were designed to amplify tet^r & strep resistance

1) Blunt in PCK against at 5' end.

Neomycin/spectinomycin Resistance Marker
Sequence Range: 1 to 1429

```

10  20  30  40  50  60  70
ATCCTAGGTCCTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCA

80  90  100 110 120 130 140
AGGCCGCAATGCCTGACGATCGGTGGAGACCGAAACCTTGCCTCGTTCGCCAGCCAGGACAGAAATGC

150 160 170 180 190 200 210
CTCGACTTCGCTGCTGCCAAGGTGGCCGGGTGACGCACACCGTGGAAACGGATGAAGGCACGAACCCAG

220 230 240 250 260 270 280
TGGACATAAGCCTGTTCCGTTTCGTAAGCTGTAATGCAAGTAGCGTATGCCCTCACGCAACTGTGCCAGAA

290 300 310 320 330 340 350
CCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTATGACTGTTTTTTGG

360 370 380 390 400 410 420
GGTACAGTCTATGCCCTCGGCCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTGCGATGTTTGATGTTATGG

430 440 450 460 470 480 490
AGCAGCAACGATGTTACGCAGCAGGCGCAGTCGCCCTAAAACAAAATTAAACATCATGAGGGAAGCGGTGA

500 510 520 530 540 550 560
TCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGCGTCATCGAGCGCCATCTCGAACCCAGCGTGTCT

570 580 590 600 610 620 630
GGCCGTACATTTGTACCGCTCCGCACTGATGGCGGCCCTGAAGCCACACAGTGATATTGATTGCTGGTT

640 650 660 670 680 690 700
ACGGTGACCGTAAAGCTTGATGAACAAACGGCGCGAGCTTTGATCAACGACCTTTTGGAAACITCGGCTT

710 720 730 740 750 760 770
CCCTGGAGAGAGCGAGATTCTCCGGCTGTAGAAGTCACCATTTGTGTGCAGCAGCATCATTCGGTG

780 790 800 810 820 830 840
GCGTTATCCAGCTAAGCGGAAGTCAATTTGGAGAAATGGCAGCGCAATGACATTCTTGCAGGTATCTTC

850 860 870 880 890 900 910
GAGCCAGCCAGGATCGACATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGG

920 930 940 950 960 970 980
TAAGTCCAGCGCGGAGGAAGTCTTGATCCGGTTCTGAAACAGGATCTATTGAGGCGCTAAATGAAAC

990 1000 1010 1020 1030 1040 1050
CTTAACGCTATGGAAGTCCGCCGCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCGCCG

1060 1070 1080 1090 1100 1110 1120
ATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTGCTGCCGACTGGGCAATGGAGCGCC

1130 1140 1150 1160 1170 1180 1190
TGCCGGCCAGTATCAGCCCGCATACTTGAAGCTAGACAGGCTTATCTTGACAAAGAAGAAGATCGCTT

1200 1210 1220 1230 1240 1250 1260
GGCCTCGCGCGCAGATCAGTTGGAAGAAATTTGTCCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGC

1270 1280 1290 1300 1310 1320 1330
AAATAATGTCTAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACCTAACGCGTTAGATGCA

1340 1350 1360 1370 1380 1390 1400
CTAAGCACATAATTGCTCACAGCCAACTATCAGGTCAAGTCTGCTTTTATTATTTTAAAGCGTGCAATAA

1410 1420
TAAGCCCTACACAAATTGGGAGATATATC

```

Bla I site

This will allow for unique
Markers in each
plasmid for storage of
synthesis - provide HTP
stock method with less
background.

Page No. 4

From Page No. 3

Spect seq

forward

1281

1429

reverse

imers for strep/spec. resistance

sulting fragment is 1281 bp in length

gtagggttattatgcagct reverse Strep_Rev
 agaaatgcctcgacttgcct forward2 Strep_Fw

Add 5' ...

Use pKOS173-176 as template

1) Blunt in PCR fragment at EcoRV site. EcoRV site
 present in tet^r gene so cannot use here

2) New to eliminate 3' HindIII site

Tet resistance marker

1480 1490 1500 1510 1520 1530 1540
 CAGCCCCATACGATATAAGTTGTAATTCTCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGTAG

>Tet_resistance_marker

1550 1560 1570 1580 1590 1600 1610
 TTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAAATGCGCTCATCGTCATC

1620 1630 1640 1650 1660 1670 1680
 CTCGGCACCGTCACCGCTGGATGCTGTAGGCATAGGCTTGTTATGCCGGTACTGCCGGGCTCTGCGGG

1690 1700 1710 1720 1730 1740 1750
 ATATCGTCCATTCCGACAGCATCGCCAGTCACATATGGCGTGTCTAGCGCTATATGCGTTGATGCAATT

1760 1770 1780 1790 1800 1810 1820
 TCTATGCGCACCGGTTCTCGGAGCACTGTCCGACCGCTTTGGCCGCCGCCAGTCTGCTCGCTTCGCTA

1830 1840 1850 1860 1870 1880 1890
 CTTGGAGCCACTATCGACTACGGATCATGGCGACACACCCGCTCTGGGATCCTCTACGCCGAGACGCA

1900 1910 1920 1930 1940 1950 1960
 TCGTGCCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCTATATCGCCGACATCACCGATGGGGA

1970 1980 1990 2000 2010 2020 2030
 AGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTCGGCGTGGGTATGGTGGCAGGCCCGGTGGCC

2040 2050 2060 2070 2080 2090 2100
 GGGGGACTGTTGGGGCGCATCTCCTTGCATGCACCATTCCTTGGCGCGCGGTGCTCAACGGCTCAACC

2110 2120 2130 2140 2150 2160 2170
 TACTACTGGGCTGCTTCTCTAATGCAGGAGTGCATAAAGGAGAGCGTCGACCGATGCCCTTGAGAGCCTT

2180 2190 2200 2210 2220 2230 2240
 CAACCCAGTCAGCTCCTTCGGTGGCGCGGGCATGACTATCGTCGCGCACTTATGACTGTCITCTIT

2250 2260 2270 2280 2290 2300 2310
 ATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGGGTCAATTTTCGGCGAGGACCGCTTTCGCTGGA

2320 2330 2340 2350 2360 2370 2380
 GCGGACGATGATCGGCTGTGCTTGGGTAATTCGGAATCTTGCAAGCCCTCGCTCAAGCCTTCGTAC

2390 2400 2410 2420 2430 2440 2450
 TGGTCCCGCCACAAACGTTTCGGCGAGAAGCAGGCCATTATCGCCGGCATGGCGGCCGACGCGCTGGGC

2460 2470 2480 2490 2500 2510 2520
 TACGCTTGTGCTGGGTTCCGCGACGCGAGGCTGGATGGCTTCCCAATTATGATTCTTCTCGCTTCGGCG

2530 2540 2550 2560 2570 2580 2590
 GCATCGGGATGCCGCGTTGACAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCA

2600 2610 2620 2630 2640 2650 2660
 AGGATCGCTCGCGGCTCTTACCAGCCTAACTTCGATCACTGACCGCTGATGTCACGGCGATTATGCG

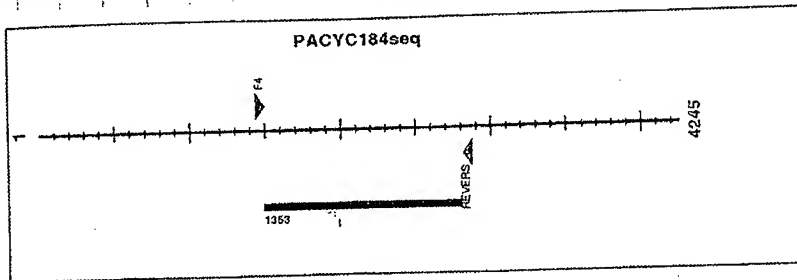
2670 2680 2690 2700 2710 2720 2730
 GCCTCGGCGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCGCCCTATACCTTGTCTGCCTCCCG

2740 2750 2760 2770 2780 2790 2800
 CGTTGCGTCGCGTGCATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTAACGGA

2810 2820 2830 2840 2850 2860 2870
 TTCACCACTCCAAGAATTGGAGCCAATCAATTCITGCGGAGAATGTGAATGCGCAACCAACCTTGGC

2880 2890 2900 2910 2920 2930 2940
 AGAACATATCCATCGCGTCCGCCATCTCCAGCAGCCGACGCGGCGCATCTCGGGCAGCGTTGGGTCTT

From Page No.



PRIMERS for Tet resistance:
Resulting fragment is 1353 bp

GCTCCAATTCTTGGAGTGGT
GCCCCATACGATATAAGTTG

Tet - Rev
REVERSE
F4 Tet - For

ORDER # 6179684
CUSTOMER # 90122
P.O. # 14509

Oligonucleotide Data Sheet

Seq #	Seq Name	Seq 5' to 3'	OD	pmol	Len	MW	µg	E260	Tm	Scale	Purif.
11502	Tet_For	GCCCCATACGATATAAGTTG	14.06	71525.12	20	6101.05	436.38	196574.3	58.35	50 nmole	Salt-Free
11503	Tet_R	GCTCCAATTCTTGGAGTGGT	10.76	58037.96	20	6139.08	356.3	185395.9	60.4	50 nmole	Salt-Free
11504	Strep_For	CCTCAGAAATGCCTCGACTTCGCT	13.71	63236.84	24	7248.77	458.39	216804	66.28	50 nmole	Salt-Free
11505	Strep_Rev	GTAGGGCTTATTATGCACGCT	11.78	59144.53	21	6452.29	381.62	199173.1	60.61	50 nmole	Salt-Free

200 pmol/µL stock
20 pmol/µL worky

Set up PCR

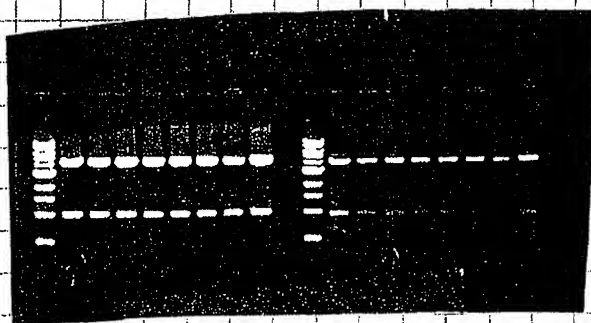
0.5 Vent polymerase
1 µL 10mM dNTPs
5 µL 10x PCR buffer
0.75 MgSO₄
1 µL Primer (Tet on Strep 20 pmol/µL)
1 µL template
40 µL water

PCR : 95 2m but 1 could not work,
25x { 95 30sec see primer at all.
54 45sec run out primer
72 1min 30
72 10min

sample template

1 1:10 pBR322
2 1:20 "
3 1:10 pAY1109
4 1:20 "



om Page No. 16

For pKOS399-53 + strep = pKOS399-16-69
pKOS399-52 + tet = pKOS399-16-~~78~~78

Site Directed Mutagenesis of 399-16-69 (strep/kan/carb)

Destroy BfuA I site in strep gene—design oligos for Quikchange (stratagene)

Changing GCAGGT (Ala Gly) to GCTGGT at position 899 in the vector

399-17-strep_sdm_for

5' GCGCAATGACATTCTTGCTGGTATCTTCGAGCCAGCC 3'

399-17-strep_sdm_rev

5' GGCTGGCTCGAAGATAACCAGCAAGAATGTCATTGCGC3'

Site Directed Mutagenesis of 399-16-78 (tet/cm/carb)

Destroy BfuA I site in tet gene ---design oligos for Quikchange (stratagene)

Changing agGCAGGTa (Arg Gln Val) to agGCAAGTa at position 1285 in vector

399-17-tet-sdm_for

5' GCAGGCCATGCTGTCCAGGCAAGTAGATGACGACC 3'

399-17-tet-sdm_rev

5' GGTCGTCATCTACTTGCTGGACAGCATGGCCTGC 3'

Cancel this
copy
BfuA I
15.10.06
Good
[Signature]

To Page No. _____

From Page No. _____

Primers for dU Cloning of Synthons:**Universal Amplification Primers**

Currently we are using

316-4-For_Morph_dU:

5' GCUAUAUCGCUAUCGAUGAGCUGCCACTGAGCACCAACTACG 3'

316-4-Rev_Morph_dU:

5' GCUAGUGAUCGAUGCAUUGAGCUGGCACTTCGCTCACTACACC 3'

However, now that we are using the new type II's restriction sites we want to add a better
Need to have Forward have Bsa I site (5' GGTCTC3') and reverse to have Bbs I (5'
GAAGAC3') site and a stuffer base (since cuts N2/N6)

So new primers to test will be:

Forward: 399-57-dU_For

5' GCUAUAUCGCUAUCGAUGAGCUGCCACTGAGCACAGGGTCTC 3'

*AG before Bsa I site was changed from CA to prevent methylation problems

Reverse: 399-57-dU_Rev

5' GCUAGUGAUCGAUGCAUUGAGCUGGCACTTCGCTCACGAAGACC 3'

*last C is stuffer base, but it cannot be a T, as it would cause methylation problems when
Xba I site present in synthon.

Blue present in dU cloning vector.

RED is Modified T7 primer that is universal at 5' of all synthons

GREEN is modified T3 primer that is universal at 3' end of all synthons

PURPLE is the Bsa I site

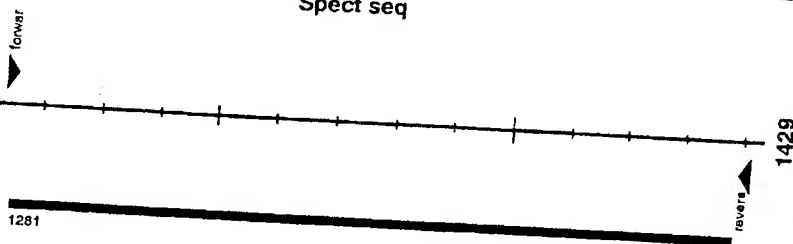
ORANGE is the Bbs I site

To Page No. _____

EXHIBIT 4

From Page No. 3

Spect seq



imers for strep/spec. resistance

sulting fragment is 1281 bp in length

gtagggtattatgcacgct reverse Strep_Rev
 agaaatgctcgacttgcct forward2 Strep_Fw

Add 5' C overhang

Use pKOS173-176 as template

1) Blunt in PstI fragment at EcoRV site EcoRV site
 present in tet^r gene no overhang

2) Need to eliminate BlnAI site

Tet resistance marker
 1480 1490 1500 1510 1520 1530 1540
 CAGCCCCATACGATATAAGTTGTAATTCTCATGTTTACAGCCTATCATCGATAAOCITTAATGGGGTAG

5' Tet resistance marker
 1550 1560 1570 1580 1590 1600 1610
 TTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAATGGGCTCATGTCATC

1620 1630 1640 1650 1660 1670 1680
 CTCGGCACCGTCACCGTGGATGCTGTAGGCATAGGCTGGTTATGCGGGTACTGCCGGGCTCTTGGGG

1690 1700 1710 1720 1730 1740 1750
 ATATCGTCCATTCCGACAGCATGCCAGTCACATATGGGTGCTGCTAGCGCTATATGCGTTGATGCAATT

1760 1770 1780 1790 1800 1810 1820
 TCTATGCGCACCGTTCTCGGAGCACTGTCCGACCGCTTGGCGCGGCCAGTCTGCTCGCTTCGCTA

1830 1840 1850 1860 1870 1880 1890
 CTTGGAGCCACTATCGACTACCGATCATGGCGACCCACACCGTCTCTGGGATCTCTACGCCGACGCA

1900 1910 1920 1930 1940 1950 1960
 TCGTGGCGGCATCACCGGCGCCACAGGTGGGTTGCTGGCGCTATATCGCGGACATCACCGATGGGGA

1970 1980 1990 2000 2010 2020 2030
 AGATCGGGCTCGCCACTTCGGGCTCATGAGCGTTGTTTCGGCGTGGGTATGGTGGCAGGCGCCGTCGCC

2040 2050 2060 2070 2080 2090 2100
 GGGGAGCTGTTGGCGGCATCTCTTGCATGCAACCATCTCTGGCGGCGGCTGCTCAACGGCTCAACC

2110 2120 2130 2140 2150 2160 2170
 TACTACTGGGCTGCTTCTCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCCCTGAGAGCCTT

2180 2190 2200 2210 2220 2230 2240
 CAACCCAGTCAGCTCTTCCGTTGGCGCGGGGCTGACTATCGTCCCGCACTTATGACTGCTCTTCTT

2250 2260 2270 2280 2290 2300 2310
 ATCATGCAACTCTTAGGACAGGTGCCGCGAGCGCTCTGGGTCAATTTTCGGCGAGGACCGCTTCCCTGGA

2320 2330 2340 2350 2360 2370 2380
 GCGCGAGATGATCGGCTGTGCTTGGGTATTCGGAATCTTGACGCGCTCGCTCAAGCCTCTCTCAC

2390 2400 2410 2420 2430 2440 2450
 TGGTCCCGCCACAAACGTTTCGGCGAGAAACAGGCCATTATCGCGCGATGGCGGCGGACCGCTGGGC

2460 2470 2480 2490 2500 2510 2520
 TACGCTTCTGCGGCTTCGGACCGGAGGCTGGATGGGCTTCCCAATTATGATTCTTCTCGCTTCGGCG

2530 2540 2550 2560 2570 2580 2590
 GCATCGGGATGCCCGGTTGACAGGOCATCTGTCCAGGCAGGTAGATGACGACCATCAGGACAGCTTCA

2600 2610 2620 2630 2640 2650 2660
 AGGATCGCTCGCGCTCTTACGAGCCTAACTTCGATCACTGGACCGCTGATGTCAGGCGGATTATGCC

2670 2680 2690 2700 2710 2720 2730
 GCGTCCGCGAGCACATGGAAACGGTTGGCATGGATTGTAGGCGCGCGCTATACCTTGTCTGCTCCCGC

2740 2750 2760 2770 2780 2790 2800
 CGTTGCGTCCGCTGCATGGAGCGCGGCCACCTCGACCTCAATGGAAGCGCGGCACTCTGCTAACGGA

2810 2820 2830 2840
 TGGAGCCAATCAATTCTTGGGAGAACTGTGAATGCGCAACCAACCCCTTGGC

2910 2920 2930 2940
 CCGCCATCTCAGCAGCGCAACCGCGGCATCTCGGCGAGCGTTGGGTCTT

Module Assembly From Synthons

Synthesis of each PKS module gene (~ 5,000 bp) requires the stitching together of about ten 500 bp synthons in a specific order. Conventionally this would require gel-purification of the individual synthons before ligation, and careful analysis of the product to make certain the new synthon has been introduced. Here, we describe an approach to circumvent this that utilizes a selectable marker to identify and discriminate the product from the original accepting vector. If successful, it should be possible to perform the ligations without purifying the synthon fragments, and the procedure would only require plasmid purifications after each round of synthon insertion.

The idea is to create two different vectors into which individual synthons (e. g. 1 through 12) are initially cloned (Figure 2). There are restriction sites (A, B, C, etc.) at both the left and right edges of the synthons (A-1-B, B-2-C, C-3-D, etc.). The two vectors would share a common selectable marker (e.g. SM1), and each would have a second distinct marker (SM2 or SM3) downstream of the synthon insert. Between the second marker and SM1 would be a "right" restriction site, R, and between SM1 and the left edge of the insert would a "left" restriction site L or L', different for each vector. Synthons 1, 4, 6, 7 etc. are be cloned into the vector with the SM2 marker, and 2, 3, 5, 8 etc. would be cloned into the vector with the SM3 marker. The initial association of specific synthons (depending on their position in the module) with SM2 or 3 is important for downstream cloning steps.

The SM's in various vectors are as follows:

EXHIBIT 5

From Page No. 1

Testing 4 Piece ligation to see what

Samples for 4-piece ligation test with and without blunt cutter

1. Digest Samples

A 6 uL of DNA 034-03 (Cm/Tet) 1 uL Bbs I 1 uL Xho I 3 uL 10 x BSA 3 uL buffer 2 16 water (to 30)	6 uL of DNA 034-04 (Kan/Strep) 1 uL Bbs I 1 uL Bsa I 3 uL 10 x BSA 3 uL buffer 2 16 water (to 30uL)	6 uL of DNA 034-05 (Cm/Tet) 1 uL Bbs I 1 uL Bsa I 3 uL 10 x BSA 3 uL buffer 2 16 water (to 30)	6 uL of DNA 034-06 (Kan/Strep) 1 uL Bsa I 1 uL Xho I 3 uL 10 x BSA 3 uL buffer 3 16 water (to 30uL)
B 6 uL of DNA 034-04 1 uL Bbs I 1 uL Xho I 3 uL 10 x BSA 3 uL buffer 2 16 water (to 30)	6 uL of DNA 034-05 1 uL Bbs I 1 uL Bsa I 3 uL 10 x BSA 1 uL Stu I 3 uL buffer 2 15 water (to 30uL)	6 uL of DNA 034-06 1 uL Bbs I 1 uL Bsa I 3 uL 10 x BSA 1 uL EcoRV 3 uL buffer 2 15 water (to 30)	6 uL of DNA 034-07 1 uL Bsa I 1 uL Xho I 3 uL 10 x BSA 3 uL buffer 3 15 water (to 30uL)

2. Verify digest on gel (1/20 inactive 20 uL 034-05)

3. Set up T4 o/n and quick ligations

4. Transform and select Kan/Tet



Set up T4 a quick ligation

3 uL 034-04 #93
 2 uL 034-05 A2 (Bbs/Bsa)
 3 uL 034-06 A6 (Bbs/Bsa)
 2 uL 034-07
 3 uL ligation buffer
 17 uL H₂O
 1.5 uL

1/2 quick
 3
 2
 3
 2
 10 uL H₂O
 1.5 uL

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